RESULTS
RESULTS

DENATURATION OF CYT-C BY DIFFERENT DENATURING AGENTS AT pH 7.0 AND 25 ± 0.05 °C.

GdnHCl Denaturation of Cyt-c:

Figure 4 shows the difference absorbance spectra of cyt-c in the presence of various concentrations of GdnHCl. The reference in each case was the native protein in the 0.03 M cacodylic acid and 0.1 M KCl, pH 7.0. For clarity only some spectra have been presented. The peak observed in each spectrum was read to calculate $\Delta\varepsilon$, difference molar extinction coefficient which is plotted as a function of GdnHCl concentration in Figure 5A; it has been observed that the position of the peak is around 403 nm. It can be seen in this figure that the change in $\Delta\varepsilon_{403}$ in the range 0-1.6 M GdnHCl is linear in the denaturant concentration. As can be seen from the circular dichroism measurements, to be described later, this change is assumed to represent the solvent effect. Above 1.60 M denaturation starts and is over at around 3.20 M above which increase in $\Delta\varepsilon_{403}$ is considered as the solvent effect on the denatured protein.

The dependence of $y_N$ was obtained from the results obtained in the pretransition region 0-1.60 M. The function describing this dependence obtained by the least-squares analysis is given by the relation.
FIGURE 4

Typical difference spectra of cyt-c produced upon denaturation by varying concentrations of GdnHCl in the visible region (500-350 nm) at 25°C and pH 7.0. (1) 0.60 M, (2) 2.00 M, (3) 2.40 M, (4) 2.80 M and (5) 3.20 M GdnHCl.
FIGURE 5

Changes in $\Delta \varepsilon_{403}$ of cyt-c on denaturation by GdnHCl. (A) transition curve, (B) normalized transition curve, (C) variation of $\Delta G_{\text{app}}$ as a function of GdnHCl concentration. Experimental conditions are the same as in Figure 4.
where \([\text{GdnHCl}]\) is the molar concentration of GdnHCl. Similarly the dependence of \(y_D\) on the denaturant concentration was obtained from the data in the posttransition region. This dependence is described by the relation

\[
y_D = 24130 (\pm 256) + 1297 (\pm 57) [\text{GdnHCl}] \quad \ldots (15)
\]

The value of 24130 M\(^{-1}\)cm\(^{-1}\) for \(y_D\) is the difference spectral property of the hypothetical denatured protein in the buffer.

Figure 5B shows the normalized transition curve in terms of \(f_D\) versus GdnHCl concentration. From the values of \(f_D\) in the range 0.1 to 0.9 equilibrium constant \(K_{\text{app}}\) and hence \(\Delta G_{\text{app}}\), the free energy change on the denaturant were calculated using eqs 7 and 8. Values of \(\Delta G_{\text{app}}\) were plotted against the molar concentration of denaturant shown in Figure 5C. A least-squares analysis according to the eq 13 gave values of \(m\) and \(\Delta G_{\text{app}}^{\text{H}_2\text{O}}\) which are listed in Table II.

The same GdnHCl induced transition was also followed by observing changes in the difference spectral intensity in the near UV-region. Typical spectra of the protein in the presence of denaturant versus native protein are shown in Figure 6. From these spectral measurements \(\Delta A_{280}\) were determined and plotted against GdnHCl concentration in Figure 7A. It is seen in this figure that the property \(y_N\) does not show any dependence on the denaturant concentration. In the posttransition region a positive solvent effect was
TABLE II: Thermodynamic Parameters Characterizing the GdnHCl and Urea Denaturation of Cytochrome-c at 25 ± 0.05 °C and pH 7.0.

<table>
<thead>
<tr>
<th>Denaturant</th>
<th>$\Delta G_{H_2O}^{\text{app}}$, kcal mol^{-1}</th>
<th>m, kcal/molmol^{-1}</th>
<th>$C_m$, M</th>
</tr>
</thead>
<tbody>
<tr>
<td>GdnHCl (VIS)</td>
<td>$6.85 \pm 0.12$</td>
<td>$2.70 \pm 0.05$</td>
<td>2.54</td>
</tr>
<tr>
<td>GdnHCl (UV)</td>
<td>$6.46 \pm 0.33$</td>
<td>$2.62 \pm 0.14$</td>
<td>2.47</td>
</tr>
<tr>
<td>urea (VIS)</td>
<td>$8.13 \pm 0.15$</td>
<td>$1.26 \pm 0.02$</td>
<td>6.45</td>
</tr>
<tr>
<td>urea (UV)</td>
<td>$8.04 \pm 0.21$</td>
<td>$1.17 \pm 0.03$</td>
<td>6.87</td>
</tr>
</tbody>
</table>
Near-UV difference spectra of cyt-c produced upon denaturation by varying concentrations of GdnHCl at 25°C and pH 7.0. (1) 1.76 M, (2) 2.40 M, (3) 3.20 M, (4) 4.00 M and (5) 6.40 M GdnHCl.
FIGURE 7

Changes in $\Delta \varepsilon_{280}$ of cyt-c on denaturation by GdnHCl. (A) transition curve, (B) normalized transition curve and (C) variation of $\Delta G_{\text{app}}$ as a function of GdnHCl concentration.
observed. This solvent effect can be calculated from the equation

\[ y_D = -13000 (\pm 80) + 506 (\pm 17) [\text{GdnHCl}] \quad \text{(16)} \]

which was obtained from the results above 3.20 M GdnHCl concentration. A value of \(-13000 (\pm 80) \text{ M}^{-1}\text{cm}^{-1}\) for \(\Delta e_{290}\) was observed for the hypothetical denatured state of cyt-c in the absence of denaturant. Results of Figure 7A were converted into a \(f_D\) shown in Figure 7B. Equations 7 and 8 were used to calculate \(K_{\text{app}}\) and \(\Delta G_{\text{app}}\) respectively.

Figure 7C shows the plot of \(\Delta G_{\text{app}}\) versus GdnHCl concentration. A least-squares analysis of the results according to eq 13 yielded values of \(m\) and \(\Delta G_{H_2O}^{\text{app}}\) which are listed in Table II.

**Urea Denaturation of Cyt-c:**

Figure 8A represents a plot of the value of the \(\Delta e_{405}\) against the molar concentration of urea. It can be seen in this figure that pretransition region is between 0 to 3.50 M above which transition region starts. In the transition region between 3.50 to 8.20 M there is an equilibrium between native and denatured protein. The linear increase from 8.20 M to 10.50 M is the posttransition region. All the points in the pretransition region were used to determine the dependence of \(y_N\) on the urea concentration. The relation
FIGURE 8

Changes in $\Delta \varepsilon_{405}$ of cyt-c on denaturation by urea. (A) transition curve, (B) normalized transition curve and (C) variation of $\Delta G_{\text{app}}$ as a function of urea.
y_N = 605 (± 16) [urea] ...(17)

The relation describing dependence of y_D on urea concentration was obtained from all the values of y_0& in the region 8.20-10.50 M. The relation observed is given by the equation

y_D = 24330 (± 94) + 321 (± 10) [urea] ...(18)

This value of 24330 (± 94) M^{-1} cm^{-1} for y_D is the value for the hypothetical denatured state in the buffer.

The normalized transition curve obtained with the help of eq 6, is shown in Figure 8B. The values of equilibrium constant and free energy change were calculated using eqs 7 and 8. Figure 8C shows the plot of ΔG_{app} versus urea concentration. A least-squares analysis of the results shown in this figure using eq 13 yielded values of ΔG_{app}, m, and C_m which are listed in Table II.

The transition curve shown in Figure 8A is a plot of Δε_{290} as a function of urea concentration. The peak at 290 nm was selected because it is mainly due to the exposure of tryptophan and heme. It can be seen in this figure that the change in Δε_{290} is zero up to 3.50 M, that is, y_N = 0.0 at all urea concentrations. Addition of urea to the cyt-c solutions above 3.50 M causes a sigmoidal decrease in the
difference molar extinction coefficient of the protein up to 8.30 M. The region between 3.50 to 8.30 M is the transition region. Addition of urea to the denatured protein causes a linear increase in the absorption property. This increase in the region from 8.30 to 10.50 M is the solvent perturbation of the exposed tryptophan and heme in the denatured protein. A linear least-squares analysis of all data in the region 8.30-10.50 M gave the dependence of \( y_D \) on the urea concentration. The equation thus obtained is

\[
y_D = -13224 \pm 384 + 1100 \pm 43 \text{[urea]} \quad \ldots (18)
\]

These informations were used in the determination of the normalized curve shown in Figure 9B which shows a plot of \( f_D \) as a function of urea concentration. Values of \( f_D \) were obtained with the help of eq 6.

\( f_D \) values were converted into \( \Delta G_{\text{app}} \) values using eqs 7 and 8. They are shown in Figure 9C as a function of urea concentration. A least-squares analysis of these results according to eq 13 yielded parameters, \( \Delta G_{\text{H}_2\text{O}} \), \( m \) and \( C_m \) which are listed in Table II.

**LiCl Denaturation of Cyt-c:**

Isothermal denaturation of cyt-c by LiCl was followed by observing changes in the difference spectral intensity in both VIS and UV regions. Difference absorbance values at 399 nm at which peak occurs were used in calculating \( \Delta \varepsilon \). Figure 10A shows a plot of \( \Delta \varepsilon_{399} \) as a
FIGURE 10

Changes in $\Delta \varepsilon_{399}$ of cyt-c on denaturation by LiCl. (A) transition curve, (B) normalized transition curves, (I): N ↔ X, (II): X ↔ D, and (C) variation of $\Delta G_I$ and $\Delta G_{II}$ as a function of LiCl concentration.
function of LiCl. It can be seen in this figure that the difference molar absorbance coefficient increases linearly with an increase in LiCl concentration from 0 to 4.0 M which is the pretransition region. A least-squares analysis was applied to determine the dependence of \( y_N \) on the LiCl concentration using all the points in the pretransition region. The relation thus obtained is as follows

\[
y_N = 1322 \pm 61 \, [\text{LiCl}]
\]

Above 4.0 M sigmoidal increase occurs up to 6.1 M where the value of optical property is 24000 \( \text{M}^{-1} \text{cm}^{-1} \), a value observed for both GdnHCl and urea denatured protein in the buffer. It is interesting to note that at LiCl concentrations above 6.1 M \( \Delta \varepsilon \) decreases with an increase in the denaturant concentration. The linear decrease above 7.84 M was found to be described by a straight line

\[
y_D = 24311 \pm 682 - 1076 \pm 76 \, [\text{LiCl}]
\]

This equation was obtained by the method of least-squares using all values in the range 7.84-10.80 M LiCl concentration. Here again the value of \( y_D \) is 24311 \( \pm 682 \) \( \text{M}^{-1} \text{cm}^{-1} \) in the absence of denaturant. Results shown in Figure 10A clearly suggest that the LiCl denaturation of cyt-c involves at least two stages. For the discussion to follow the first stage (stage I) will be called a transition between
N and X, the intermediate state and the second stage (stage II) will represent a transition between X and D states.

For the stage I, i.e. reaction, $N \leftrightarrow X$, values of $y_N$ at different concentrations of LiCl were calculated from eq 20 and for the X state a value of 24000 M$^{-1}$cm$^{-1}$ was assumed to be independent of denaturant concentration. These informations were used in the determination of the values of $f_I$ with the help of eq 9. A plot of $f_I$ as a function of LiCl concentration is shown in Figure 10B.

For the stage II, i.e., the reaction $X \leftrightarrow D$, the normalized transition curve was obtained by plotting $f_{II}$ as a function of LiCl concentration. Values of $f_{II}$ at each concentration were obtained using eq 10. $y_D$ values were obtained from eq 21. Apparent free energy changes, $\Delta G_I$ and $\Delta G_{II}$ for transitions $N \leftrightarrow X$ and $x \leftrightarrow D$, respectively, were calculated using eqs 11 and 12. These were plotted against the LiCl concentrations in Figure 10C. For both stages a least-squares analysis according to eq 13 was used to calculate all thermodynamic parameters that are entered in Table III.

$\Delta \varepsilon_{290}$ values were determined at different concentrations of LiCl from the difference spectra. A plot of $\Delta \varepsilon_{290}$ against the denaturant concentration is shown in Figure 11A. As can be seen in this figure there is no change in difference spectral intensity in the 0-4.0 M concentration range. Change in the environment of tryptophan and heme occurs above 4.0 M, and this change seems to be complete at about 7.84 M above which a linear increase occurs. This was
TABLE III: Thermodynamic Parameters Characterizing the Denaturation of Cytochrome-c Under Various Denaturant Conditions at 25 ± 0.05°C and pH 7.0.

**PHASE I**

<table>
<thead>
<tr>
<th>Denaturant</th>
<th>$\Delta G_{12}^{H_2O}$, kcal mol$^{-1}$</th>
<th>$m$, kcal/mol mol$^{-1}$</th>
<th>$c_m$, M</th>
</tr>
</thead>
<tbody>
<tr>
<td>LiCl (VIS)</td>
<td>6.74 (± 0.32)</td>
<td>1.73 (± 0.05)</td>
<td>5.05</td>
</tr>
<tr>
<td>LiCl (UV)</td>
<td>7.12 (± 0.28)</td>
<td>1.46 (± 0.06)</td>
<td>4.88</td>
</tr>
<tr>
<td>LiBr (VIS)</td>
<td>7.67 (± 0.72)</td>
<td>2.24 (± 0.21)</td>
<td>3.42</td>
</tr>
<tr>
<td>LiBr (UV)</td>
<td>6.40 (± 0.30)</td>
<td>1.92 (± 0.09)</td>
<td>3.33</td>
</tr>
<tr>
<td>LiClO$_4$ (VIS)</td>
<td>6.26 (± 0.38)</td>
<td>4.16 (± 0.27)</td>
<td>1.51</td>
</tr>
<tr>
<td>NaBr (VIS)</td>
<td>7.85 (± 0.31)</td>
<td>1.26 (± 0.05)</td>
<td>6.23</td>
</tr>
<tr>
<td>CaCl$_2$ (VIS)</td>
<td>8.31 (± 0.18)</td>
<td>3.55 (± 0.08)</td>
<td>2.43</td>
</tr>
<tr>
<td>CaCl$_2$ (UV)</td>
<td>6.97 (± 0.08)</td>
<td>3.04 (± 0.04)</td>
<td>2.29</td>
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</table>

**PHASE II**

<table>
<thead>
<tr>
<th>Denaturant</th>
<th>$\Delta G_{II}^{a}$, kcal mol$^{-1}$</th>
<th>$m$, kcal/mol mol$^{-1}$</th>
<th>$c_m$, M</th>
<th>$\Delta G_{12}^{H_2O}$, kcal mol$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>LiCl (VIS)</td>
<td>1.94 (± 0.05)</td>
<td>2.13 (± 0.06)</td>
<td>6.95</td>
<td>10.68</td>
</tr>
<tr>
<td>LiCl (UV)</td>
<td>1.68 (± 0.32)</td>
<td>1.80 (± 0.35)</td>
<td>7.01</td>
<td>8.81</td>
</tr>
<tr>
<td>LiBr (VIS)</td>
<td>1.22 (± 0.14)</td>
<td>1.12 (± 0.12)</td>
<td>5.07</td>
<td>8.89</td>
</tr>
<tr>
<td>LiBr (UV)</td>
<td>1.55 (± 0.07)</td>
<td>1.19 (± 0.05)</td>
<td>5.32</td>
<td>7.85</td>
</tr>
<tr>
<td>LiClO$_4$ (VIS)</td>
<td>1.82 (± 0.22)</td>
<td>4.68 (± 0.60)</td>
<td>2.19</td>
<td>8.08</td>
</tr>
<tr>
<td>CaCl$_2$ (VIS)</td>
<td>1.46 (± 0.07)</td>
<td>4.05 (± 0.18)</td>
<td>3.26</td>
<td>9.77</td>
</tr>
<tr>
<td>CaCl$_2$ (UV)</td>
<td>1.66 (± 0.08)</td>
<td>1.88 (± 0.08)</td>
<td>3.78</td>
<td>6.63</td>
</tr>
</tbody>
</table>

$\Delta G_{II}^{a}$ represents the value obtained from extrapolation of $\Delta G_{II}$ values in the pretransition region of the process $X \leftrightarrow D$. 
FIGURE 11

Changes in $\Delta \varepsilon_{290}$ of cyt-c on denaturation by LiCl. (A) transition curve, (B) normalized transition curve, (I): $N \leftrightarrow X$, (II): $X \leftrightarrow D$, and (C) variation of $\Delta G_1$ and $\Delta G_2$, as a function of LiCl concentration.
taken as solvent effect on the $\Delta \varepsilon_{290}$. This solvent effect was found to be described by the equation

$$y_D = -13078 (\pm 322) + 653 (\pm 35) [\text{LiCl}] ...(22)$$

It can be seen in Figure 11A that the whole process is divided into two transitions, namely $N \leftrightarrow X$ and $X \leftrightarrow D$. This division is based on our measurements of $\Delta \varepsilon_{399}$ values shown in Figure 10A. Assuming that $y_X$ is independent of LiCl concentration and assigning a value of $-7280$ M$^{-1}$ cm$^{-1}$ for $y_X$, we calculated $f_I$ values at each concentration. Figure 11B shows the plot of $f_I$ versus LiCl concentration. Transition $X \leftrightarrow D$ was analyzed using eq 10. In the calculation of $f_{II}$, $y_X$ was assumed to be independent of the concentration of LiCl and values of $y_D$ at different concentrations of the denaturant were determined from eq 22.

The values of the fractional change for transitions I and II were converted into $\Delta G_I$ and $\Delta G_{II}$ values using eqs 11 and 12, respectively. Plot of these free energy changes are shown in Figure 11C. Each plot were analyzed using eq 13. All thermodynamic parameters obtained from the least-squares analysis are given in Table III.

It is interesting to note that $f$ values as well as $\Delta G$ values determined from two different properties fall on the same curve for each transition (results not shown here). This appears to justify our analysis of the transition curve obtained from observing changes from $\Delta \varepsilon_{290}$. Furthermore our assumption that transitions $N \leftrightarrow X$ and $X \leftrightarrow D$ represent two
two-state transitions seems to be correct.

LiBr Denaturation of Cyt-c:

A plot of $\Delta \varepsilon_{399}$ versus LiBr concentration is shown in Figure 12A. There occurs a linear increase in optical property up to 2.50 M LiBr concentration. This linear increase is the solvent effect which can be defined by the relation

$$y_N = 1996 (\pm 155) [\text{LiBr}]$$  \hspace{1cm} \text{...(23)}$$

With the increase in LiBr concentration from 2.50 M to 4.0 M a sigmoidal increase in optical property occurs which represents stage I. The value of $\Delta \varepsilon$ at 4.0 M LiBr is 24000 M$^{-1}$cm$^{-1}$. This is the value of $y_X$. With the further increase in LiBr concentration above 4.0 M $\Delta \varepsilon$ value decreases. There occurs a linear decrease in the region 6.50-9.00 M. The latter is taken as the solvent perturbation of the denatured protein and is called posttransition region. The dependence of $y_D$ is described by the equation

$$y_D = 24072 (\pm 554) - 1501 (\pm 73) [\text{LiBr}]$$  \hspace{1cm} \text{...(24)}$$

obtained from the least-squares analysis of all the values of $\Delta \varepsilon_{399}$ in the posttransition region. It can be seen in eq 24 that $\Delta \varepsilon_{399}$ value of 24072 (± 554) is obtained at zero molar LiBr concentration.

Assuming that $y_X$ is independent of LiBr
FIGURE 12

Changes in $\Delta \varepsilon_{399}$ of cyt-c on denaturation by LiBr. (A) transition curve, (B) normalized transition curve, (I): N $\leftrightarrow$ X, (II): X $\leftrightarrow$ D, and (C) variation of $\Delta G_I$ and $\Delta G_{II}$ as a function of LiBr concentration.
concentration, values of \( f_1 \) were determined using eq 9. Similarly values of \( f_{II} \) at different concentrations of denaturant were estimated using eq 10 but values of \( y_D \) at any concentration of denaturant was determined from eq 24. Figure 12B shows plots of \( f_1 \) and \( f_{II} \) against [LiBr].

Values of \( AG_1 \) and \( AG_{II} \) were determined from \( f \) values using, respectively, eqs 11 and 12. The free energy changes in the presence of LiBr for both transitions are shown in Figure 12C. The thermodynamic parameters, given in Table III, for both the stages I and II were calculated according to the eq 13.

From the difference absorbance spectra of cyt-c in the presence of varying concentrations of LiBr values of \( \Delta \varepsilon_{290} \) were read. Figure 13A shows a plot of \( \Delta \varepsilon_{290} \) versus [LiBr]. There appears no change in the pretransition region (i.e., \( \gamma_N = 0.0 \)). Optical property decreased from 2.50 M to 6.50 M above which a linear increase in \( \Delta \varepsilon \) values occurs which is the posttransition region solvent effect. By using a least-squares analysis the dependence of \( y_D \) can be described by the equation as follows

\[
y_D = -13106 (\pm 275) + 593 (\pm 35) [\text{LiBr}] \quad \ldots (25)
\]

Here again the transition curve was divided in two transitions based on \( \Delta \varepsilon_{399} \) measurements shown in Figure 12A. A value of \(-7400 \text{ M}^{-1} \text{ cm}^{-1}\) was obtained for \( \gamma_X \) which was assumed to be independent of the denaturant concentration. From the known values of \( \gamma_X \) and \( y_D \), \( f_1 \) and \( f_{II} \) were determined
Changes in $\Delta\varepsilon_{280}$ of cyt-c on denaturation by LiBr. (A) transition curve, (B) normalized transition curve, (I): N $\leftrightarrow$ X, (II): X $\leftrightarrow$ D, and (C) variation of $\Delta G_1$ and $\Delta G_{II}$ as a function of LiBr concentration.
with the help of eqs 9 and 10. Figure 13B shows the normalized curves, which were obtained by plotting $f_I$ and $f_{II}$ against the LiBr concentration. Free energy changes, $\Delta G_I$ and $\Delta G_{II}$ for both the stages were calculated using the eqs 11 and 12, respectively. These free energy changes, plotted as a function of LiBr concentration, are shown in Figure 13C. Equation 13 was used to analyze the thermodynamic parameters given in Table III.

**LiClO$_4$ Denaturation of Cyt-c:**

As observed for LiCl and LiBr, cyt-c denaturation by LiClO$_4$ also produces two distinct stages in visible region (see Figure 14A). A linear increase from 0 M to 0.80 M in the pretransition region is the solvent effect which could be best described by the relation

$$y_N = 8573 \pm 1500 \text{ [LiClO}_4]$$  ... (26)

As can be seen in this figure, $\Delta \varepsilon_{405}$ increases with the increase in LiCO$_4$ concentration upto 1.80 M, where a $\Delta \varepsilon$ value of 24000 M$^{-1}$ cm$^{-1}$ is obtained. After this, decrease in $\Delta \varepsilon$ values occurred with an increase in the denaturant concentration. After 2.40 M LiCO$_4$ concentration a linear decrease occurs. The later effect is considered as solvent effect on the denatured protein. A least-squares analysis of all the values of $\Delta \varepsilon_{405}$ in the posttransition region yielded the [LiClO$_4$] dependence of $y_D$ which is described by the relation
FIGURE 14

Changes in $\Delta \varepsilon_{405}$ of cyt-c on denaturation by LiClO$_4$ (A) transition curve, (B) normalized transition curve, (I): N $\Leftrightarrow$ X, (II): X $\Leftrightarrow$ D, and (C) variation of $\Delta G_I$ and $\Delta G_{II}$ as a function of LiClO$_4$ concentration.
\[ y_D = 24089 (\pm 937) - 783 (\pm 302) [\text{LiClO}_4] \quad (27) \]

A \( \Delta \varepsilon \) value of \( 24089 (\pm 937) \text{ M}^{-1} \text{ cm}^{-1} \) at zero molar \( \text{LiClO}_4 \) is in very well agreement with the one obtained by GdnHCl, urea, LiCl and LiBr induced unfolding of the cyt-c.

The normalized transition curves shown in the Figure 14B were obtained by plotting \( f_I \) and \( f_{II} \) values against the concentration at which they were calculated, with the help of eqs 9 and 10. In the determination of \( f_I \) a value of \( 24000 \text{ M}^{-1} \text{ cm}^{-1} \) for \( y_X \) was assumed to be independent of \( [\text{LiClO}_4] \). Value of \( y_D \) in presence of various concentrations of denaturants were determined using eq 27. Equilibrium constants and subsequently the Gibbs free energy changes were calculated from \( f_I \) and \( f_{II} \) using eqs 11 and 12. Figure 14C shows the plot of \( \Delta G_I \) and \( \Delta G_{II} \) values as a function of \( \text{LiClO}_4 \) concentration. A least-squares analysis was used to fit the data according to eq 13 to calculate all thermodynamic parameters presented in Table III.

We have attempted to measure the transition following changes in the difference spectral property of cyt-c in the near-UV region (results not shown). It has been observed that \( \Delta \varepsilon_{290} \) did not change in the region 0-0.80 M \( \text{LiClO}_4 \) concentration. At concentrations above 0.80 M there is a decrease in the optical property, but this decrease was not very sigmoidal. Hence we did not attempt to analyze these results for \( \Delta G_{\text{H}_{2}\text{O}} \) values.
NaBr denaturation of Cyt-c:

Isothermal denaturation of cyt-c by NaBr was followed by observing changes in the difference spectral intensity in visible region. Figure 15A shows a plot of $\Delta \varepsilon_{401}$ as a function of [NaBr]. It is evident from this figure that there is a linear increase in difference molar absorbance coefficient from 0 to 4.0 M. This region is assumed to be the pretransition region. All the points in the pretransition region were used to define the solvent effect. The relation thus obtained is as follows:

$$y_N = 237 \pm 100 \text{ [NaBr]} \quad \ldots (28)$$

Above 4.0 M sigmoidal increase occurs but the transition is not complete even in the presence of highest concentration of the denaturant. Assuming that this reaction corresponds to the transition $N \leftrightarrow X$, we have used a value of 24000 $\text{M}^{-1}\text{cm}^{-1}$ for $y_X$ which was taken as independent of NaBr concentration. We have used this value of $y_X$ on the basis of our observations that in all the denaturants used to unfold the cyt-c a value of 24000 $\text{M}^{-1}\text{cm}^{-1}$ independent of denaturant concentration was obtained. These informations were used in the determination of the values of $f_I$ with the help of eq 9. A plot of $f_I$ as a function of NaBr concentration is shown in Figure 15B.

Apparent free energy changes, $\Delta G_I$ for transition $N \leftrightarrow X$ (stage I) were calculated using eq 11. Figure 15C shows
Changes in $\Delta absorptivity$ of cyt-c on denaturation by NaBr. (A) transition curve, (B) normalized transition curve and (C) variation of $\Delta G^\circ$ as a function of NaBr concentration.
a plot of $\Delta G_1$ as a function of [NaBr]. A least-squares analysis according to eq 13 was used to calculate all thermodynamic parameters that are listed in Table III.

CaCl$_2$ Denaturation of Cyt-c:

The values of $\Delta \varepsilon$ at the peaks of the CaCl$_2$ induced difference absorbance spectra of cyt-c in the visible region were read in order to construct the transition curve shown in Figure 16A. CaCl$_2$ also induced conformational changes in two steps. It is seen in Figure 16A that the conformational changes appear to start above 1.50 M. A slight increase in $\Delta \varepsilon_{405}$ from 0 to 1.50 M represents a solvent effect on the protein. The equation used to define pretransition region solvent effect is

$$y_N = 1567 (\pm 89) [\text{CaCl}_2] \quad ...(29)$$

The increase in optical property occurred up to 2.90 M CaCl$_2$ where $\Delta \varepsilon$ value of 24000 M$^{-1}$ cm$^{-1}$ is obtained. Here again a decrease in optical property occurred. From 4.25 M CaCl$_2$ concentration onward the decrease becomes linear which is considered as posttransition region. The equation used to define the posttransition solvent effect is

$$y_D = 24000 (\pm 308) - 3032 (\pm 68) [\text{CaCl}_2] \quad ...(30)$$

The normalized transition curves obtained by
Changes in $\Delta \varepsilon_{405}$ of cyt-c on denaturation by CaCl$_2$:

(A) transition curve, (B) normalized transition curves, (I): $N \rightarrow X$, (II): $X \rightarrow D$, and (C) variation of $\Delta G_I$ and $\Delta G_{II}$ as a function of CaCl$_2$ concentration.
plotting $f_I$ and $f_{II}$ against CaCl$_2$ concentration are shown in Figure 16B. These were calculated according to the eqs 9 and 10. In the determination of $f_I$ it was assumed that $y_X$ (24000 M$^{-1}$ cm$^{-1}$) is independent of the concentration of CaCl$_2$. With the help of eqs 11 and 12 $\Delta G_I$ and $\Delta G_{II}$ were calculated. A plot of $\Delta G_I$ and $\Delta G_{II}$ as a function of CaCl$_2$ concentration is shown in Figure 16C. Table III shows all thermodynamic parameters obtained from the analysis of the free energy changes versus [CaCl$_2$] plots according to eq 13.

Figure 17A shows a plot of $\Delta \varepsilon_{280}$ versus CaCl$_2$ concentration. It is seen that there is no change in optical property from 0 to 1.50 M CaCl$_2$ (i.e. $y_N = 0.0$). Above 1.50 M CaCl$_2$ concentration $\Delta \varepsilon$ values starts decreasing with an increase in concentration up to 4.25 M CaCl$_2$. There is a linear increase in the $\Delta \varepsilon$ values above 4.25 M CaCl$_2$. The equation used to define the solvent effect on the optical property of the denatured protein is

$$y_D = -12980 (\pm 566) + 1889 (\pm 124) [\text{CaCl}_2] \ldots (31)$$

As discussed for the lithium salts induced transitions in near UV-region, here also the transition curve was divided into two transitions (see Figure 17A). The division was based on our visible region studies (Figure 16A). Figure 17B shows the normalized transition curves obtained by plotting $f_I$ and $f_{II}$ as a function of the CaCl$_2$ concentration, with the help of eqs 9 and 10.

Using eqs 11 and 12 the free energy changes $\Delta G_I$
FIGURE 17

Changes in $\Delta \varepsilon_{290}$ of cyt-c on denaturation by CaCl$_2$ (A) transition curve, (B) normalized transition curves, (I): $I \Leftrightarrow X$, (II): $X \Leftrightarrow D$, and (C) variation of $\Delta G_I$ and $\Delta G_{II}$ as a function of CaCl$_2$ concentration.
and $\Delta G_{II}$ were calculated. A least-squares analysis of the
data according to the eq 13 yielded the thermodynamic
parameters presented in Table III.

**Acid Denaturation of Cyt-c:**

Difference in the absorption of cyt-c at pH 5.0 and
that at other pH values was measured in the visible region
(500 to 350 nm). From these measurements values of $\Delta \varepsilon$ at the
peak which is around 395 nm were read and plotted in the
Figure 18A. It can be seen that there is a linear increase in
the difference spectral intensity in the pH range 5 to 4
below which unfolding starts and is complete at pH 1.37. It
has been observed that $y_N$ can be described by the relation

$$y_N = -1989 \pm 121 \text{ pH} + 9833 \pm 541 \quad \ldots (32)$$

and $y_D$ is assumed to be independent of pH ($y_D = 75250 \text{ M}^{-1}
\text{ cm}^{-1}$).

These observations were used to analyze the
transition curve using eqs 6-8. The values of $f_D$ as a
function of pH are shown in Figure 18B. The midpoint of
transition is around 2.3 in 0.05 M Gly-HCl buffer containing
0.01 M KCl. Figure 18C shows a plot between $\Delta G_{app}$ calculated
from eq 8 and pH. Results above pH 2 suggest that there are
about 1.5 more protons bound to the unfolded protein. Since
pH-dependence of the $\Delta G_{app}$ is not known, the stability of the
cyt-c at pH cannot estimated.
FIGURE 18

Changes in $\Delta \varepsilon_{395}$ of cyt-c on denaturation by variation of pH values (A) transition curve, (B) normalized transition curve and (C) variation of $\Delta G_{\text{app}}$ as a function of pH values.
CD MEASUREMENTS:

The denaturation transition of cyt-c was followed by measuring far-UV CD spectra of the protein in presence of different denaturants. From these measurements values of \( [\theta]_{222} \) were read and are plotted in Figure 19. All the transitions shown in this figure appear to be very cooperative. It has been observed that the cyt-c denaturation by GdnHCl, LiCl and pH is reversible. All denaturants gave the same value for \( [\theta]_{222} \) of the denatured protein.
FIGURE 19

Changes in ellipticity of cyt-c on denaturation by GdnHCl (1), LiCl (2), and pH (3) (F. Ahmad, unpublished results).
**Viscosity Measurements:**

Figure 20 shows the plot of $\eta_{\text{red}}$ of the native and GdnHCl-denatured cyt-c as a function of protein concentration. A least-squares analysis of these results according to eq 3 yielded values of 2.67 ($\pm$ 0.23) and 14.61 ($\pm$ 0.19) ml/g for the values of intrinsic viscosity of cyt-c in the native and GdnHCl denatured conformations. It has been observed that the values of Huggins constant, $k$ of the native protein decreases from 2.62 to 0.64 in presence of 3.5 M GdnHCl in which the cyt-c is known to behave as random coil (Privalov et al., 1989).

In order to characterize the end products of reaction N $\leftrightarrow$ X and N $\leftrightarrow$ D values of $[\eta]$ in presence of other denaturants namely LiCl, LiBr, LiClO$_4$, and CaCl$_2$ were also measured. Figure 21 shows the plots of $\eta_{\text{red}}$ versus [protein]. A least-squares analysis according to the relation 3 was used to fit the data. The values of $[\eta]$ and $k$ obtained in presence of these denaturants are listed in Table IV.
Reduced viscosity of ferricytochrome-c. (○) Buffer and (□) 3.50 M GdnHCl.
FIGURE 21

Intrinsic viscosity measurements of the intermediate (X) and randomly coiled (D) states obtained in different denaturants.
TABLE IV: Intrinsics Viscosity of Cytochrome-c Under Various Solvent Conditions at 25 ± 0.05 °C and pH 7.0.

<table>
<thead>
<tr>
<th>solvent</th>
<th>$[\eta]$, ml/g</th>
<th>Huggins constant, $k$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>2.67 ± .23</td>
<td>2.62</td>
</tr>
<tr>
<td>GdnHCl</td>
<td>14.61 ± .19</td>
<td>0.64</td>
</tr>
<tr>
<td>urea$^a$</td>
<td>14.90</td>
<td>0.60</td>
</tr>
<tr>
<td>LiCl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(phase I)</td>
<td>3.49 ± .04</td>
<td>6.64</td>
</tr>
<tr>
<td>(phase II)</td>
<td>14.53 ± .16</td>
<td>1.00</td>
</tr>
<tr>
<td>LiBr</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(phase I)</td>
<td>3.96 (± .22)</td>
<td>11.44</td>
</tr>
<tr>
<td>(phase II)</td>
<td>14.84 (± .16)</td>
<td>0.79</td>
</tr>
<tr>
<td>LiClO$_4$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(phase I)</td>
<td>3.24 (± .46)</td>
<td>30.39</td>
</tr>
<tr>
<td>(phase II)</td>
<td>14.48 (± .46)</td>
<td>1.77</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(phase I)</td>
<td>3.60 (± .11)</td>
<td>19.68</td>
</tr>
<tr>
<td>(phase II)</td>
<td>14.62 (± .13)</td>
<td>0.93</td>
</tr>
</tbody>
</table>

$^a$ Taken from Stellwagen (1968).
GdnHCl induced denaturation in presence of various concentrations of different salts was followed by measuring $\Delta \varepsilon$ in the visible region. The raw data were converted into transition curves from which the midpoint of transition and changes in Gibbs free energies of stability of cyt-c were obtained using appropriate equations, given in the Materials and Methods section. Figures 22 to 34 are divided in four parts A, B, C and D which show respectively, the transition curves, normalized curves, variations in $c_m$ and Gibbs free energy change with the denaturant concentration.

For the quantitative analysis of the denaturation data the solvent effect functions were first defined. The equations used to define the solvent effect were derived with the assumption that the solvent effects were linearly dependent upon the denaturant concentration. The points clearly outside the transition region were used in a least mean squares analysis to define the pre- and posttransition regions solvent effects. These informations were used in the determination of the $f_D$ versus GdnHCl plot, also known as normalized transition curve.

Lithium Chloride:

Figure 22A shows the GdnHCl induced transition curves in presence of 0.90, 1.75, 2.58, and 4.35 M LiCl. The pretransition region at all concentrations of LiCl is best
FIGURE 22


dinHCl denaturation of cyt-c in presence of 0 (-), 0.90 (O), 1.75 (○), 2.58 (△) and 4.35 (●) M LiCl. (A) transition curves, (B) normalized transition curves, (C) variation in $C_m^S$ values and (D) variation in $ΔG_{app}$ values.
defined by the relation,

\[ y_N = 1380 (\pm 170) [\text{GdnHCl}] + 1320 (\pm 40) [\text{LiCl}] \quad \ldots (33) \]

It is evident from the eq 33 that \( y_N \) depends both on [GdnHCl] and [LiCl]. The equations used to define the posttransition solvent effect are

\[ y_D = 24050 (\pm 190) + 290 (\pm 50) [\text{GdnHCl}] \quad \ldots (34) \]

at 0.90, 1.75, and 4.35 M LiCl, and

\[ y_D = 23610 (\pm 250) + 1360 (\pm 80) [\text{GdnHCl}] \quad \ldots (35) \]

at 2.58 M LiCl.

It is evident from transition curves shown in Figure 22A that LiCl destabilizes the protein. Normalized curves are shown in Figure 22B. Figure 22C shows the plot of \( C_m^S \) as a function of [LiCl]. \( C_m^S \) value decreases with an increase in the concentration of LiCl.

Figure 22D shows the plots of the change in Gibbs energy against [GdnHCl]. \( \Delta G_{\text{app}}^{H_2O,s} \) value decreases with an increase in the LiCl concentration. All thermodynamic parameters are given in Table V.

Lithium Bromide:

Figure 23A shows the GdnHCl induced transition
TABLE V: Thermodynamic Parameters Characterizing the Denaturation of Cytochrome-c by GdnHCl in Presence of Various Concentrations of Different Salts.

<table>
<thead>
<tr>
<th>SALTS, (M)</th>
<th>$\Delta G_{H_2O,s}^{\circ}$ (kcal mol$^{-1}$)</th>
<th>$m_g^s$ (kcal mol/mol$^{-1}$)</th>
<th>$C_m^s$ (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LiCl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.90 M</td>
<td>6.14 ± .23</td>
<td>2.67 ± .10</td>
<td>2.30</td>
</tr>
<tr>
<td>1.75 M</td>
<td>5.54 ± .10</td>
<td>2.71 ± .05</td>
<td>2.04</td>
</tr>
<tr>
<td>2.58 M</td>
<td>3.02 ± .17</td>
<td>1.97 ± .12</td>
<td>1.53</td>
</tr>
<tr>
<td>4.35 M</td>
<td>1.10 ± .09</td>
<td>2.72 ± .17</td>
<td>0.40</td>
</tr>
<tr>
<td>LiBr</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.00 M</td>
<td>6.64 ± .34</td>
<td>3.63 ± .18</td>
<td>1.83</td>
</tr>
<tr>
<td>1.50 M</td>
<td>5.84 ± .28</td>
<td>3.90 ± .19</td>
<td>1.50</td>
</tr>
<tr>
<td>2.00 M</td>
<td>4.25 ± .12</td>
<td>3.56 ± .10</td>
<td>1.19</td>
</tr>
<tr>
<td>2.80 M</td>
<td>1.72 ± .12</td>
<td>3.15 ± .18</td>
<td>0.55</td>
</tr>
<tr>
<td>LiClO$_4$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.20 M</td>
<td>5.43 ± .12</td>
<td>2.50 ± .05</td>
<td>2.17</td>
</tr>
<tr>
<td>0.40 M</td>
<td>2.73 ± .14</td>
<td>1.76 ± .09</td>
<td>1.53</td>
</tr>
<tr>
<td>0.80 M</td>
<td>1.16 ± .06</td>
<td>1.63 ± .06</td>
<td>0.71</td>
</tr>
<tr>
<td>SALTS,</td>
<td>$\Delta G_{H_2O,s}^{app}$ (kcal mol$^{-1}$)</td>
<td>$m$ (kcal mol/mol)</td>
<td>$n$ (M)</td>
</tr>
<tr>
<td>--------------</td>
<td>---------------------------------------------</td>
<td>-------------------</td>
<td>---------</td>
</tr>
<tr>
<td>(M)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaClO$_4$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.48 M</td>
<td>$2.96 \pm 0.13$</td>
<td>$1.73 \pm 0.08$</td>
<td>1.71</td>
</tr>
<tr>
<td>0.76 M</td>
<td>$2.35 \pm 0.09$</td>
<td>$1.37 \pm 0.09$</td>
<td>$&lt;0$</td>
</tr>
<tr>
<td>1.90 M</td>
<td>$0.97 \pm 0.03$</td>
<td>$3.14 \pm 0.09$</td>
<td>0.29</td>
</tr>
<tr>
<td>NaBr</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.40 M</td>
<td>$6.10 \pm 0.49$</td>
<td>$3.21 \pm 0.26$</td>
<td>1.90</td>
</tr>
<tr>
<td>2.10 M</td>
<td>$3.51 \pm 0.44$</td>
<td>$2.12 \pm 0.24$</td>
<td>1.67</td>
</tr>
<tr>
<td>3.15 M</td>
<td>$2.93 \pm 0.23$</td>
<td>$2.28 \pm 0.15$</td>
<td>1.29</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.50 M</td>
<td>$6.36 \pm 0.13$</td>
<td>$3.17 \pm 0.06$</td>
<td>1.35</td>
</tr>
<tr>
<td>1.00 M</td>
<td>$5.57 \pm 0.15$</td>
<td>$3.40 \pm 0.09$</td>
<td>1.64</td>
</tr>
<tr>
<td>1.40 M</td>
<td>$3.23 \pm 0.25$</td>
<td>$2.62 \pm 0.21$</td>
<td>1.23</td>
</tr>
<tr>
<td>2.25 M</td>
<td>$0.68 \pm 0.23$</td>
<td>$4.11 \pm 0.88$</td>
<td>0.17</td>
</tr>
<tr>
<td>NH$_4$Cl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.60 M</td>
<td>$7.23 \pm 0.37$</td>
<td>$3.02 \pm 0.15$</td>
<td>2.38</td>
</tr>
<tr>
<td>0.80 M</td>
<td>$7.70 \pm 0.73$</td>
<td>$3.24 \pm 0.32$</td>
<td>3.38</td>
</tr>
<tr>
<td>NaCl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.49 M</td>
<td>$7.65 \pm 0.31$</td>
<td>$3.00 \pm 0.12$</td>
<td>2.55</td>
</tr>
<tr>
<td>0.97 M</td>
<td>$7.76 \pm 0.21$</td>
<td>$3.06 \pm 0.08$</td>
<td>2.54</td>
</tr>
<tr>
<td>SALTS,</td>
<td>$\Delta G_{\text{water}}^{\text{H}_2\text{O}, s}$</td>
<td>$m_s^G$</td>
<td>$C_s^G$</td>
</tr>
<tr>
<td>----------------</td>
<td>-----------------------------------------------</td>
<td>---------</td>
<td>---------</td>
</tr>
<tr>
<td>(M)</td>
<td>(kcal mol$^{-1}$)</td>
<td>(kcal mol/mol$^{-1}$)</td>
<td>(M)</td>
</tr>
<tr>
<td>KCl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$0.49\text{ M}$</td>
<td>$6.80 \pm 0.32$</td>
<td>$2.68 \pm 0.12$</td>
<td>$2.54$</td>
</tr>
<tr>
<td>$0.86\text{ M}$</td>
<td>$7.57 \pm 0.46$</td>
<td>$2.94 \pm 0.19$</td>
<td>$2.58$</td>
</tr>
<tr>
<td>CsCl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$0.70\text{ M}$</td>
<td>$7.33 \pm 0.49$</td>
<td>$2.92 \pm 0.20$</td>
<td>$2.51$</td>
</tr>
<tr>
<td>$1.40\text{ M}$</td>
<td>$6.49 \pm 0.38$</td>
<td>$2.55 \pm 0.15$</td>
<td>$2.55$</td>
</tr>
<tr>
<td>RbCl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$0.60\text{ M}$</td>
<td>$6.99 \pm 0.66$</td>
<td>$2.71 \pm 0.27$</td>
<td>$2.58$</td>
</tr>
<tr>
<td>$1.20\text{ M}$</td>
<td>$7.21 \pm 0.31$</td>
<td>$2.76 \pm 0.12$</td>
<td>$2.61$</td>
</tr>
<tr>
<td>($\text{NH}_4$)SO$_4$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$0.40\text{ M}$</td>
<td>$7.82 \pm$</td>
<td>$2.70 \pm$</td>
<td>$2.90$</td>
</tr>
<tr>
<td>$0.60\text{ M}$</td>
<td>$8.64 \pm$</td>
<td>$2.70 \pm$</td>
<td>$3.20$</td>
</tr>
<tr>
<td>$\text{Na}_2\text{H}_2\text{PO}_4$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$0.40\text{ M}$</td>
<td>$7.25 \pm 0.43$</td>
<td>$2.38 \pm 0.14$</td>
<td>$3.05$</td>
</tr>
<tr>
<td>$0.68\text{ M}$</td>
<td>$8.96 \pm 0.13$</td>
<td>$2.65 \pm 0.04$</td>
<td>$3.38$</td>
</tr>
</tbody>
</table>
FIGURE 23

nHCl denaturation of cyt-c in presence of G (-), 1.00 (O),
0.50 (■), 2.00 (A) and 2.80 (●) M LiBr. (A) transition
curves, (B) normalized transition curves, (C) variation in $C_m^*$
values and (D) variation in $\Delta G_{app}$ values.
curves in presence of various concentrations of LiBr. From the values of $\Delta \varepsilon$ in pretransition region, the following equation was determined using a least-squares analysis:

$$2240 (\pm 90) [\text{GdnHCl}] + 2000 (\pm 160) [\text{LiBr}] \quad \text{(36)}$$

This shows that $y_N$ depends both on the concentration of GdnHCl and LiBr. As can be seen in Figure 23A, $\Delta \varepsilon$ observed in the posttransition region of denaturation curve in the presence of LiBr can be described by a single line which is given by the eq 37 which was obtained using a least-squares analysis.

$$y_D = 23770 (\pm 300) + 320 (\pm 120) [\text{GdnHCl}] \quad \text{(37)}$$

Figure 23B shows the normalized curves obtained by plotting $f_D$ values as a function of $[\text{GdnHCl}]$. The free energy change ($\Delta G_{\text{app}}$) plots are shown in Figure 23D. Least-squares analysis method was used to fit the data according to the eq 13. Thermodynamic parameters are given in Table V.

**Lithium PerChlorate:**

Figure 24A shows GdnHCl induced transition curves in presence of three different concentrations of LiClO$_4$. The equation used to define the pretransition region is

$$y_N = 2240 (\pm 91) [\text{GdnHCl}] + 8573 (\pm 1500) [\text{LiClO}_4] \quad \text{(38)}$$
FIGURE 24

HCl denaturation of cyt-c in presence of 0 (-), 0.20 (○),
0.40 (□), and 0.80 (△) M LiClO₄; (A) transition curves, (B)
normalized transition curves, (C) variation in C₅ values and
(D) variation in ΔGₚ values.
eq 38 shows the observation that \( y_N \) depends both on [GdnHCl] and [LiClO₄] (see Figure 24A).

The least-squares analysis of all the data in the posttransition region of each transition curve gave the dependence of \( y_D \) on the [GdnHCl] in the presence of a fixed concentration of LiClO₄. At 0.20 M LiClO₄

\[
y_D = 24110 \pm 180 + 280 \pm 50 \text{ [GdnHCl]} \tag{38}
\]

and at 0.40 and 0.80 M LiClO₄

\[
y_D = 24010 \pm 230 + 980 \pm 70 \text{ [GdnHCl]} \tag{40}
\]

The normalized transition curves, obtained by plotting \( f_D \) values as a function of GdnHCl concentration, are shown in Figure 24B. A plot of \( C_m^5 \) against [LiClO₄] is shown in Figure 24C. It can be seen in this figure that the \( C_m^5 \) value decreases with an increase in the concentration of LiClO₄.

Figure 24D shows the plot of free energy changes (\( \Delta G_{\text{app}} \)) versus GdnHCl concentration. A least-squares analysis of the data according to the eq 13 yielded the thermodynamic parameters that are presented in Table V.

**Sodium PerChlorate:**

Figure 25A shows the transition curves obtained by
FIGURE 25

GdnHCl denaturation of cyt-c in presence of 0 (−), 0.48 (○), 0.76 (○) and 1.90 (Δ) M NaClO₄. (A) transition curves, (B) normalized transition curves, (C) variation in $C_m^S$ values and (D) variation in $\Delta G_{app}$ values.
plotting Δr values against [GdnHCl] in presence of 0.48, 0.76 and 1.90 M NaClO₄. All Δr values in pretransition region were used in the least-squares analysis to define the solvent effect. The relation thus obtained is

\[ y_N = 3830 (± 410) [\text{GdnHCl}] + 3470 (± 120) [\text{NaClO}_4] \quad (41) \]

Equation 41 works well for all the NaClO₄ concentrations used here. It is clear from this equation that \( y_N \) depends both on the [GdnHCl] and [NaClO₄]. On the contrary \( y_D \) does not depend upon the NaClO₄ concentration in the range 0.48-1.90 M. The GdnHCl dependence of \( y_D \) in the presence of NaClO₄ is given by the relation,

\[ y_D = 24280 (± 240) + 250 (± 110) [\text{GdnHCl}] \quad (42) \]

With the solvent effects defined and with the help of eqs 41 and 42, the normalized curves were obtained by plotting \( f_D \) values against GdnHCl concentration. These transition curves are shown in Figure 25B.

Figure 25C shows the variation in \( C_m^* \) values as a function of [NaClO₄]. From the normalized transition curves the equilibrium constants and subsequently the Gibbs free energy changes were calculated according to the eq 8.

Figure 25D shows the plots of ΔG_{app} values as a function of the GdnHCl concentration. A least-squares analysis was used to analyze these data. The analysis yielded values of
\[ \Delta G_{H_2O,s}^{n}, m, S_{m} \text{ and } C_{m}^{S} \text{ which are given in Table V.} \]

**Sodium Bromide:**

GdnHCl induced unfolding transition of cyt-c in presence of three different concentrations of NaBr is shown in Figure 26A. A least-squares analysis of the pretransition region data was done to define the solvent effect. The equations used are as follows: For 1.40 and 2.10 M NaBr

\[ y_N = 2280 (\pm 110) [\text{GdnHCl}] + 2000 (\pm 1100) [\text{NaBr}] \quad \ldots(43) \]

and for 3.15 M NaBr

\[ y_N = 4140 (\pm 270) [\text{GdnHCl}] + 2000 (\pm 1100) [\text{NaBr}] \quad \ldots(44) \]

Similarly a least-squares analysis of posttransition region data was done to define the solvent effect on \( y_D \). The equations used are as follows: at 1.40 and 3.15 M NaBr

\[ y_D = 23810 (\pm 200) + 810 (\pm 60) [\text{GdnHCl}] \quad \ldots(45) \]

and at 2.10 M NaBr

\[ y_D = 24070 (\pm 390) - 130 (\pm 120) [\text{GdnHCl}] \quad \ldots(46) \]

With the help of eq 6 values of \( f_D \) were determined. The normalized curves are shown in Figure 26B.
FIGURE 26

HCl denaturation of cyt-c in presence of 0 (-), 1.40 (○), 0 (□) and 3.15 (△) M NaBr. (A) transition curves, (B) alized transition curves, (C) variation in $C_m^S$ values and variation in $\Delta G_{app}$ values.
Figure 26C shows the variation in \( c_m^s \) values as a function of [NaBr]. All thermodynamic parameters, i.e., \( \Delta G^\text{H}_2\text{O}, \Delta S^s, m^s \) and \( C_m^s \) listed in Table V were calculated by a least-squares analysis according to the eq 13 results of which are shown in Figure 26D.

**Calcium Chloride:**

GdnHCl induced transition curves in presence of various concentrations of \( \text{CaCl}_2 \) are shown in Figure 27A. The pretransition region solvent effect are defined by the relation

\[
y_N = 1860 (\pm 150) [\text{GdnHCl}] + 1570 (\pm 90) [\text{CaCl}_2] \quad \ldots (47)
\]

This equation shows that \( y_N \) depends on both GdnHCl and \( \text{CaCl}_2 \) concentrations. A least-squares analysis of the posttransition region data was done to define the solvent effect. The equations used are as follows: At 0.50 M \( \text{CaCl}_2 \)

\[
y_D = 23640 (\pm 230) + 670 (\pm 70) [\text{GdnHCl}] \quad \ldots (48)
\]

and

\[
y_D = 23950 (\pm 100) + 40 (\pm 40) [\text{GdnHCl}] \quad \ldots (49)
\]

at 1.0, 1.40 and 2.25 M \( \text{CaCl}_2 \).

Using eq 6 values of \( f_D \) were determined. The
FIGURE 27

Denaturation of cyt-c in presence of 0 (-), 0.50 (O),
( ), 1.40 (Δ) and 2.25 (●) M CaCl₂ (A) transition
rates, (B) normalized transition curves, (C) variation in Cₘ
rates and (D) variation in ΔGₐₚₜ values.
Normalized transition curves are shown in Figure 27B. Figure 27C shows the variation in $C_m^S$ values with respect to the $[CaCl_2]$. All thermodynamic parameters, i.e., $\Delta G_{app}^{H_2O,s}$, $m_g$ and $C_m^S$ listed in Table V were calculated using a least-squares analysis according to the eq 13. Gibbs free energy changes with GdnHCl concentration are shown in Figure 27D.

Ammonium Chloride:

The GdnHCl induced transitions in presence of NH$_4$Cl are shown in Figure 28A. A least mean squares analysis of the pre- and posttransition data to define the solvent effect, $y_N$ and $y_D$ in the presence of NH$_4$Cl yielded the following equations

$$y_N = 2980 (\pm 100) \ [\text{GdnHCl}]$$  \hspace{1cm} \dots \ (50)$$

and

$$y_D = 24010 (\pm 1340) + 710 (\pm 380) \ [\text{GdnHCl}]$$  \hspace{1cm} \dots \ (51)$$

Normalized curves obtained after defining the solvent effects are shown in Figure 28B. Figure 28C shows the variation in $C_m^S$ values with respect to $[NH_4Cl]$. The Gibbs free energy changes were calculated with the help of eq 8. Figure 28D shows the plot of $\Delta G_{app}$ values versus $[\text{GdnHCl}]$. Equation 13 was used to analyze the thermodynamic parameters given in Table V.
FIGURE 28

Cl denaturation of cyt-c in presence of O ( ), 0.60 ( )
0.80 ( ) M NH₄Cl. (A) transition curves, (B) normalized
transition curves, (C) variation in $G_m^{s}$ values and (D)
variation in $AG_{s; p}$ values.
Sodium Chloride and Potassium Chloride:

The unfolding transition of cyt-c induced by the addition of GdnHCl in presence of 0.49 and 0.87 M NaCl and 0.49 and 0.86 M KCl were followed by observing changes in the Δς. The data obtained in this manner were converted into transition curves.

Figures 29A and 30A present the transition curves obtained by plotting Δς values against the GdnHCl concentration. The pretransition region solvent effects, y_N, in presence of NaCl and KCl depend on the GdnHCl concentration only, and the relation can be described as follows:

\[ y_N = 1720 \pm 110 \text{ [GdnHCl]} \]  \hspace{1cm} (52)

The points clearly outside the transition region were used in a least-squares analysis to define the posttransition region solvent effect in both the cases. For the 0.49 M NaCl transition the equation used to define the posttransition solvent effect, y_D, is

\[ y_D = 23430 \pm 420 + 360 \pm 110 \text{ [GdnHCl]} \]  \hspace{1cm} (53)

and for 0.97 M NaCl

\[ y_D = 2460 \pm 540 - 560 \pm 140 \text{ [GdnHCl]} \]  \hspace{1cm} (54)
FIGURE 29

Denaturation of actin in presence of 0.49 (O), 0.97 (M) M NaCl. (A) transition curves, (B) normalized transition curves, (C) variation in $G_m^a$ values and (D) variation in $\Delta G_m$ values.
FIGURE 30

Denaturation of cyt-c in presence of 0 (-), 0.4G (O); 0.86 (©) M KCl. (A) transition curves, (B) normalized transition curves, (C) variation in $C_m^3$ values and (D) variation in $\Delta G_{mp}$ values.
Similarly, the eq 55,

\[ y_D = 23440 \pm 550 + 680 \pm 150 \text{ [GdnHCl]} \]  

defines the solvent effect, obtained from a least-squares treatment of the posttransition region data for 0.49 and 0.86 M KCl.

With the solvent effects defined normalized curves can be produced. Figures 29B and 30B present the normalized transition curves in 0.49 and 0.97 M NaCl and 0.49 and 0.86 M KCl, respectively. In these figures \( f_D \) values were calculated from eq 6.

The plot of \( C_m^5 \) values as a function of NaCl and KCl are presented in Figures 29C and 30C, respectively.

The Gibbs free energy changes were calculated using eq 8. Figures 29D and 30D present plots of \( \Delta G_{app} \) values, as a function of the GdnHCl concentration used to induce the unfolding in the presence of NaCl and KCl, respectively. The linear extrapolation method was used for the estimation of \( \Delta G_{app}^{H_2O,0.5} \) values. All thermodynamic parameters obtained on the basis of eq 13 are listed in Table V.

**Cesium Chloride and Rubidium Chloride:**

Figures 31A and 32A show the GdnHCl induced transition curves in presence of 0.70 and 1.40 M CsCl and
FIGURE 31

Denaturation of cyt-c in presence of 0 (-), 0.70 (O) 1.40 (x), M CaCl. (A) transition curves, (B) normalized transition curves, (C) variation in $C_m$ values and (D) variation in $\Delta G_{app}$ values.
FIGURE 32

Cl denaturation of cyt-c in presence of 0 (-), 0.60 (O) 
and 1.20 (a) M RbCl. (A) transition curves, (B) normalized 
transition curves, (C) variation in $C_m^S$ values and (D) 
variation in $\Delta G_{\text{app}}$ values.
0.60 and 1.20 M RbCl, respectively, obtained by plotting the 
$\Delta\epsilon$ values against GdnHCl concentration. The points clearly 
outside the transition region were used in a least-squares 
analysis to define the pre- and posttransition region's 
solvent effect. The equation used to define the pretransition 
solvent effect, $y_N$, at all concentrations of both CsCl and 
RbCl is

$$y_N = 1880 \pm 130 \ [\text{GdnHCl}]$$ 

The equation used to define the posttransition solvent effect, 
$y_D$, at all concentrations of CsCl and RbCl is

$$y_D = 2370 \pm 1230 + 510 \pm 350 \ [\text{GdnHCl}]$$

It is evident from the eqs 56 and 57 that the $y_N$ and $y_D$
depend on the GdnHCl concentration only, that is, they are 
independent of the CsCl and RbCl concentrations.

Figure 31B and 32B show the normalized transition 
curves, obtained by plotting $f_D$ values against [GdnHCl]. The 
$f_D$ values were calculated according to the eq 6. Variation in 
$C_m^S$ values as a function of CsCl and RbCl is shown, 
respectively, in Figures 31C and 32C. The plot of $AG_{app}$ 
values, as a function of GdnHCl concentration is shown in 
Figures 31D and 32D. A least-squares analysis was used to fit 
the data according to the eq 13. All the thermodynamic 
parameters obtained are listed in Table V.
Ammonium Sulphate:

GdnHCl induced unfolding transition of cyt-c in presence of 0.40 and 0.60 M \((\text{NH}_4)_2\text{SO}_4\) is shown in Figure 33A. The equations used to define the pre- and posttransition regions solvent effects, \(y_N\) and \(y_D\) were as follows:

\[
y_N = 2360 (\pm 130) [\text{GdnHCl}] \quad \ldots (58)
\]

and

\[
y_D = 23830 (\pm 900) + 440 (\pm 210) [\text{GdnHCl}] \quad \ldots (59)
\]

It is evident from eq 58 that \(y_N\) is independent of \((\text{NH}_4)_2\text{SO}_4\) at all concentrations. On the other hand, the GdnHCl dependence of \(y_D\) which is same at 0.40 and 0.60 M \((\text{NH}_4)_2\text{SO}_4\), is different from that given by eq 15.

Figure 33B shows the normalized transition curves obtained by plotting \(f_D\) values versus [GdnHCl]. Equation 6 was used to calculate the \(f_D\) values. The variation in \(C_m^5\) values as a function of \([((\text{NH}_4)_2\text{SO}_4)]\) is shown in Figure 33C. Free energy changes \(\Delta G_{\text{app}}\), as a function of GdnHCl concentration are shown in Figure 33D. A least squares analysis was used to fit the data using eq 13. The values of \(\Delta G_{\text{app}}^{H_2O,s}\), \(m_g^5\) and \(C_m^5\), obtained from the least-squares analysis according to the eq 13 are listed in Table V.
Figure 33

Cl denaturation of cyt-c in presence of 0 (●), 0.40 (○) 0.60 (□) M (NH₄)₂SO₄. (A) transition curves, (B) alized transition curves, (C) variation in C₃₉ values and variation in ΔGₚₚ values.
The unfolding transition of cyt-c induced by GdnHCl in presence of 0.40 and 0.68 M NaH$_2$PO$_4$·H$_2$O was followed by observing changes in optical property. Figure 34A shows the transition curves obtained by plotting Δε values versus [GdnHCl]. The points clearly outside the transition region were used in a least-squares analysis to define the pre- and posttransition regions solvent effects in the presence of Sodium phosphate. The equations thus obtained are:

$$y_N = 1510 \pm 80 \ [\text{GdnHCl}]$$  \hspace{1cm} (60)$$

and

$$y_\text{c} = 2405 \pm 1580 + 2580 \ (330) \ [\text{GdnHCl}]$$  \hspace{1cm} (61)$$

The normalized transition curves, i.e. $f_D$ versus [GdnHCl] plots, obtained are shown in Figure 34B. Equation 6 was used to calculate the $f_D$ values. The variation in $C_m^3$ values with respect to [NaH$_2$PO$_4$·H$_2$O] is shown in Figure 34C. Equation 8 was used to calculate the values of Gibbs free energy changes, $\Delta G_{\text{app}}$ which are shown in Figure 34D as a function of GdnHCl concentration. A least-squares analysis of the data of this figure according to the eq 13 yielded the thermodynamic parameters namely, $\Delta G_{\text{app}}^{H_2O,3} / m^3$ and $C_m^3$ which are presented in Table V.
ICL denaturation of cyt-c in presence of 0 (-), 0.40 (○)
0.58 (□) M NaH₂PO₄. (A) transition curves, (B) normalized
transition curves, (C) variation in Cₚ₃ values and (D)
variation in ΔGₚ₃ values.
Analysis of the Results of Denaturation by Mixed-Denaturant System:

Assuming that each denaturant of the mixed denaturing system cooperates in unfolding cyt-c, we may write

\[ \Delta G_{\text{app}} = \Delta G_{\text{app}}^{\text{H}_2\text{O}} - m_g [g] - m_s [s] \]  \hspace{1cm} (62)

where \( m_g [g] \) and \( m_s [s] \) give the GdnHCl and salt contribution to the measured \( \Delta G_{\text{app}} \), and \( \Delta G_{\text{app}}^{\text{H}_2\text{O}} \) is the value \( \Delta G_{\text{app}} \) in absence of both denaturants at constant temperature and pH.

From eq 62 it follows that \( \Delta G_{\text{app}} \) for the GdnHCl denaturation can be corrected for the effect of the salt denaturant in the system. The corrected value of the free energy change, \( \Delta G_{\text{app}}^{\text{corr}} \), is defined as

\[ \Delta G_{\text{app}}^{\text{corr}} = \Delta G_{\text{app}} + m_s [s] = \Delta G_{\text{app}}^{\text{H}_2\text{O}} - m_g [g] \]  \hspace{1cm} (63)

we have treated all the mixed denaturant results according to the eq 63. Results are given in Figures 35 and 36 and the value of \( \Delta G_{\text{app}}^{\text{H}_2\text{O}} \) and \( m_g \) thus calculated are given in Table VI.
FIGURE 35

The role of denaturational free energy, corrected for the effect of LiCl (A), CaCl₂ (B), LiBr (C) and NaBr (D), on the rate of denaturation. It was assumed that there exists no specific site(s) on the protein for the denaturants.
FIGURE 36

Dependence of denaturational free energy, corrected for the effect of LiClO₄ (A) and NaClO₄ (B), on GdnHCl denaturation. It was assumed that there exists no binding site(s) on the protein for the denaturants.
### Table of Thermodynamic Parameters

<table>
<thead>
<tr>
<th>Denaturant</th>
<th>$M$ (kcal mol$^{-1}$ M$^{-1}$)</th>
<th>$\Delta G_{app}^{H_2O}$ (kcal mol$^{-1}$ M$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH</td>
<td>2.96 ($\pm$ 0.08)</td>
<td>8.75 ($\pm$ 0.13)</td>
</tr>
<tr>
<td>C$_2$H$_5$</td>
<td>3.23 ($\pm$ 0.08)</td>
<td>8.69 ($\pm$ 0.12)</td>
</tr>
<tr>
<td>C$_2$H$_4$</td>
<td>2.24 ($\pm$ 0.22)</td>
<td>6.97 ($\pm$ 0.37)</td>
</tr>
<tr>
<td>C$<em>{10}$H$</em>{16}$</td>
<td>1.55 ($\pm$ 0.15)</td>
<td>2.91 ($\pm$ 0.22)</td>
</tr>
<tr>
<td>Br</td>
<td>3.56 ($\pm$ 0.06)</td>
<td>8.74 ($\pm$ 0.09)</td>
</tr>
<tr>
<td>C$<em>{10}$H$</em>{14}$</td>
<td>1.79 ($\pm$ 0.05)</td>
<td>4.55 ($\pm$ 0.08)</td>
</tr>
</tbody>
</table>